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ORIGINAL PAPER

Antifungal activity of a novel chromene dimer

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Abstract The activity on *Aspergillus* spp. growth and on ochratoxin A production of two novel chromene dimers (**3**) was evaluated. The results of the bioassays indicate that the chromene dimer **3a** inhibited mycelia growth by approximately 50% (EC_{50}) at 140.1 μmol L^{-1} for *A. niger*, 384.2 μmol L^{-1} for *A. carbonarius*, 69.1 μmol L^{-1} for *A. alliaceus* and 559.1 μmol L^{-1} for *A. ochraceus*. When applied at concentrations of 2 mmol L^{-1}, **3a** totally inhibited the growth of all *Aspergillus* spp. tested. Furthermore, ochratoxin A production by *A. alliaceus* was reduced by about 94% with a 200 μmol L^{-1} solution of this compound. A moderate inhibitory effect was observed for the analogous structure **3b** on ochratoxin A production but not in mycelia growth. No inhibition was registered for compounds **2a** and **2b**, used as synthetic precursors of the dimeric species **3**.

Keywords Chromenes · *Aspergillus* spp. · Ochratoxin A · Antifungal compounds

Introduction

The screening for new antifungal chemicals is a constant need, due to the public demand for crop protection agents with low use rates, a benign environmental profile, and low toxicity to humans and wildlife. There is also a need for compounds with novel modes of action and improved efficiency, capable to combat pathogens with resistance or reduced sensitivity [10].

The use of fungicides aims at the inhibition of fungal growth and ultimately at the prevention of agricultural commodities deterioration. Nevertheless, when fungi are present the mycotoxins issue is also very important. Recent reports correlate the application of some fungicides with an increase in mycotoxin contents. For example, it was reported that azoxystrobin increased the production of deoxynivalenol per unit of pathogen in an artificially inoculated field trial [16], that miconazole and fenpropimorph increased the aflatoxin production from *A. parasiticus* in laboratory conditions [7] and that fenhexamid, mancozeb and a mixture of copper salts increased the ochratoxin A (OTA) production by *A. carbonarius* in grapes [3].

Ochratoxin A is one of the mycotoxins which is frequently reported to be present in crops. It has nephrotoxic, teratogenic, hepatotoxic, immunosuppressive and carcinogenic properties, and it is produced by some *Aspergillus* and *Penicillium* species on some specific agricultural commodities. For example, *A. carbonarius* is mainly responsible for ochratoxin A contamination in grapes [15], *A. alliaceus* for contamination in figs [2], *A. ochraceus* and *A. carbonarius* for contamination in green coffee beans [14], *P. verrucosum* in cereal grains [13] and *P. nordicum* in meat and cheese [12]. For this reason, OTA is usually found in food products such as breakfast cereals, coffee, cocoa products, dried vine fruits, dried figs, beer and wine.

Chromene derivatives are an important class of compounds, widely present in plants, including edible vegetables and fruits [6]. The biological activity of some natural chromene-based structures led to the development of synthetic analogues, some of them displaying remarkable

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effects as pharmaceuticals [1, 4, 5, 9, 11, 17], including antimicrobial agents [8].

In the present work, we tested the activity of two novel chromene dimers on *Aspergillus* spp. growth and on its ochratoxin A production. The antifungal activity of compounds used as precursors of the dimeric species was also evaluated.

Materials and methods

Compound synthesis

The (2-amino-3-cyano-4H-chromene-4-yl)malononitrile **2a** and **2b** were synthesized from the corresponding substituted salicylaldehyde and malononitrile (two molar equivalents), in an aqueous $NaHCO_3$ 0.05 mol L^{-1} solution, at room temperature (Scheme 1). The products were isolated in 91 to 100% yield after 5–35 min. The chromene dimer **3a** was synthesized from malononitrile and salicylaldehyde in a 1:1 molar ratio, using methanol as solvent and triethylamine catalysis at room temperature. The yellow solid was isolated in 85% yield after 1 day. The chromene dimer **3b** was generated from the reaction of malononitrile and 3-methoxysalicylaldehyde in a 1:1 ratio, using methanol as solvent and two drops of triethylamine. The solid mixture isolated after 6 h at room temperature was stirred in dimethyl sulfoxide (DMSO) solution for a further 21 days and isolated in 76%.

Compounds characterization

All compounds were characterized by elemental analysis, melting point and IR spectroscopy (Table 1). Elemental analysis was performed on a LECO CHNS-932 instrument. Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected. The IR spectra were obtained with an FT-IR Bomem-MB104 spectrophotometer using Nujol mulls and NaCl plates.

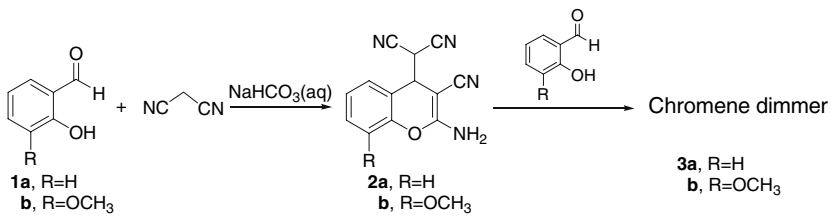
Bioassay of fungicidal activities

Biological material

Fungi used were *Aspergillus alliaceus* strain MUM 03.55, *A. carbonarius* strain MUM 03.59, *A. niger* strain MUM 03.58 and *A. ochraceus* strain MUM 03.56; all preserved in the Micoteca da Universidade do Minho (MUM) culture collection. The *A. alliaceus*, *A. carbonarius* and *A. ochraceus* strains are OTA producers, whereas the *A. niger* strain is not [15].

In vitro assays

The antifungal activity of synthesized compounds was evaluated at various concentrations by the poisoned food technique using YES medium (2% yeast extract from Difco, 15% sucrose and 2% agar). Stock working solutions of the compounds were prepared in DMSO and aseptically



Scheme 1 General synthetic route for compounds 2 and 3

Table 1 Physical properties and elemental analysis of compounds **2** and **3**

Compound	*R*	Mp (°C)	Yield (%)	IR data (cm^{-1}) (Nujol mull)	Formula	Analysis calculated (found, %)		
						C	H	N
2a	H	152–153	91.3	2,195(s), 2,144(w) 1,644(s), 1,615(w), 1,603(m), 1,576(s)	$C_{13}H_8N_4O{\cdot}0.1H_2O$	66.1 (66.0)	3.4 (3.5)	23.7 (23.7)
2b	OCH_3	168–169	99.8	2,253(w), 2,192(s) 1,642(s), 1,614(s), 1,602(s), 1,583(s)	$C_{14}H_{10}N_4O_2{\cdot}0.1H_2O$	63.2 (63.1)	3.7 (3.8)	21.1 (20.9)
3a	H	165–168	84.5	2,225(m), 2,196(s) 1,668(m), 1,650(s), 1,610(m), 1,578(m)	$C_{20}H_{12}N_4O_2{\cdot}0.1H_2O$	70.2 (70.2)	3.6 (3.6)	16.4 (16.6)
3b	OCH_3	150–152	75.7	2,227(m), 2,189(s) 1,642(s), 1,602(m), 1,585(m)	$C_{22}H_{16}N_4O_4.C_2H_6SO{\cdot}1/2H_2O$	59.14 (59.18)	4.72 (5.03)	11.50 (11.74)

added to the autoclaved culture medium to reach 50, 100 and 200 μmol L^{-1}, before plating 20 mL in 9 cm petri dishes. Culture media supplemented with DMSO were used as control. Strains were first grown in MEA medium (Blakeslee formula) for 7 days at 25 °C in the dark for inoculum generation. Spore suspensions were prepared in 1 mL of semi solid agar (0.2% agar and 0.05% Tween 80), and its concentrations were adjusted to 2.5×10^6 spores mL^{-1}, using a NeuBauer chamber. Plates were centrally inoculated with 10 μL of each spore suspension and incubated at 25 °C in the dark. All experiments were in triplicate for each compound against each fungus. Fungal colony diameters were recorded daily. Growth rates were calculated by linear regression of colony diameters against days. The concentration of active ingredient at which survival was 50% (EC_{50}) was determined by fitting the experimental data to a four-parameter logistic model (Hill equation) using computer curve-fitting software (Prism 4, GraphPad Software, Inc, San Diego, CA, USA). A microplate assay was also prepared to determine the concentration of **3a** necessary to totally inhibit spore germination of the tested strains. In a sterile 96-well microplate, assays with 0 (controls) to 2,000 μmol L^{-1} of **3a**, in 200 μL of YES medium previously inoculated with each strain, were prepared in duplicate. The microplate was incubated at 25 °C, in the dark, for 6 days, and a visual observation was done each day to record the presence of mycelia. To evaluate fungal growth in each well, different values were given: a value of 1 to wells totally covered with fungal mycelia, 0.5 when just half of the well was covered, and 0.25 when ¼ of the well was covered.

Ochratoxin A detection

The OTA produced by the ochratoxigenic strains in the experiments of the poisoned food technique was quantified after 6 days of fungal growth. Media and colonies were cut in small pieces and transferred to 50 mL Greiner tubes. Twenty milliliters of methanol were added and, after strong agitation, left to extract overnight. Three milliliters of extract was filtered through a 0.45 μm syringe filter of PTFE (Teknokroma) and 1 mL of the filtrate evaporated to dryness at 50 °C with a gentle stream of nitrogen in a clean vial. Dry extracts were resuspended in 1 mL of HPLC mobile phase and analyzed by high-performance liquid chromatography. The HPLC apparatus consisted of a Varian 9002 pump equipped with a Jasco FP-920 fluorescence detector ($\lambda_{ex} = 333$ nm; $\lambda_{em} = 460$ nm) and a Marathon Basic autosampler. The analytical column was a C_{18} reversed-phase YMC-Pack ODS-AQ (250 × 4.6 mm and 5 μm), fitted with a precolumn with the same stationary phase. The mobile phase was a mixture of acetonitrile/water/acetic acid (99/99/2, v/v/v), filtered and degassed. Flow rate was set to 0.8 mL min^{-1} and the column temperature to 30 °C; the loop volume was 100 μL. A five-point calibration curve was prepared with standards of ochratoxin A (Sigma) and regularly checked.

Statistic analysis

All statistic analyses were performed with the Statistic Package for Social Sciences (SPSS) version 15.0. Means were compared by analysis of variance followed by the Ducan's post-test.

Results

The growth rates of strains in the presence of tested compounds are summarized in Table 2. Compound **3a** was the only one to show a significant antifungal activity on all strains assessed. It was more effective on *Aspergillus alliaceus*, producing growth rates reductions from 2.05 cm day^{-1} to 1.02, 1.01 and 0.66 cm day^{-1} in the presence of 50, 100 and 200 μmol L^{-1}, respectively. *Aspergillus carbonarius* was less sensitive to **3a**. Significant reductions of growth rates from 2.22 cm day^{-1} to 2.18, 1.98 and 1.70 cm day^{-1} in the presence of 50, 100 and 200 μmol L^{-1}, respectively, were obtained for this strain. The effect of **3a** in the mycelial growth of fungi tested after 4 days of incubation can be observed in the Fig. 1. The concentration of **3a** at which survival was 50% (EC_{50}) is 140.1 μmol L^{-1} for *A. niger*, 384.2 μmol L^{-1} for *A. carbonarius*, 69.1 μmol L^{-1} for *A. alliaceus* and 559.1 μmol L^{-1} for *A. ochraceus* (Table 3). Furthermore, it was observed that 2,000 μmol $L^{-1}$of **3a** totally inhibit the spore germination of tested fungi since mycelial growth was not observed in the 96-well microplate tests, as it can be seen in Fig. 2. Lower amounts of this compound (500 and 1,000 μmol L^{-1}) increase the lag time, since visible mycelia only appear at the third and fourth day of incubation. No significant inhibition on growth rates was registered for compounds **2a** and **2b**, used as synthetic precursors of the dimeric species **3a** and **3b**.

The effect of compounds on ochratoxin A production is shown in Table 4. The production of OTA by *A. alliaceus* was significantly reduced by compounds **3a** and **3b**. In the presence of 50, 100 and 200 μmol L^{-1} of **3a**, *A. alliaceus* produced 290.1, 229.9 and 25.4 μg OTA/plate; respectively, less 32, 46 and 94% than control (427.04 μg OTA/plate). In the presence of 200 μmol L^{-1} of **3b** *A. alliaceus* produced 222.73 μg OTA/plate, less 48% than control. Significant reduction in OTA production by *A. carbonarius* was also observed with **3b**. In the presence of 200 μmol L^{-1} of **3b,** *A. carbonarius* produced 6.38 μg OTA/plate, less 61% than control (16.43 μg OTA/plate). The effect of compounds **3a** and **3b** on OTA production by *A. ochraceus* was not significant.

Table 2 Radial growth rates (cm day^{-1}) of tested strains in YES supplemented with the synthesized compounds

Compound	μmol L^{-1}	Radial growth rates (cm day^{-1})			
		A. niger	*A. carbonarius*	*A. 1alliaceus*	*A. ochraceus*
Control		2.13 ± 0.05^{a}	2.22 ± 0.03^{a}	2.05 ± 0.02^{a}	1.45 ± 0.02^{a}
3a	50	1.25 ± 0.02^{d}	2.18 ± 0.03^{b}	1.02 ± 0.05^{c}	1.33 ± 0.02^{b}
	100	1.13 ± 0.03^{e}	1.98 ± 0.04^{c}	1.01 ± 0.02^{c}	1.26 ± 0.02^{c}
	200	1.06 ± 0.03^{e}	1.70 ± 0.03^{d}	0.66 ± 0.02^{d}	1.08 ± 0.02^{d}
2a	50	1.98 ± 0.06^{b}	2.27 ± 0.02^{a}	1.98 ± 0.03^{a,b}	1.44 ± 0.01^{a}
	100	1.94 ± 0.02^{b}	2.27 ± 0.02^{a}	1.98 ± 0.01^{a,b}	1.44 ± 0.01^{a}
	200	1.80 ± 0.03^{c}	2.21 ± 0.02^{a,b}	1.86 ± 0.02^{b}	1.41 ± 0.01^{a}
3b	50	2.03 ± 0.02^{a,b}	2.17 ± 0.02^{b}	2.00 ± 0.02^{a,b}	1.41 ± 0.02^{a}
	100	2.00 ± 0.02^{a,b}	2.22 ± 0.04^{a}	1.98 ± 0.01^{a,b}	1.40 ± 0.03^{a}
	200	1.83 ± 0.05^{b,c}	2.14 ± 0.02^{b}	2.02 ± 0.04^{a,b}	1.33 ± 0.05^{b}
2b	50	2.14 ± 0.03^{a}	2.21 ± 0.02^{a,b}	2.06 ± 0.02^{a}	1.42 ± 0.02^{a}
	100	2.10 ± 0.08^{a,b}	2.20 ± 0.02^{a,b}	2.09 ± 0.02^{a}	1.44 ± 0.02^{a}
	200	2.04 ± 0.02^{a,b}	2.19 ± 0.01^{b}	2.11 ± 0.06^{a}	1.38 ± 0.03^{a,b}

Values are the mean of three replicates of each ± standard deviation (SD). Data marked with different letters are significantly different from respective control at $P < 0.001$ for the Duncan test

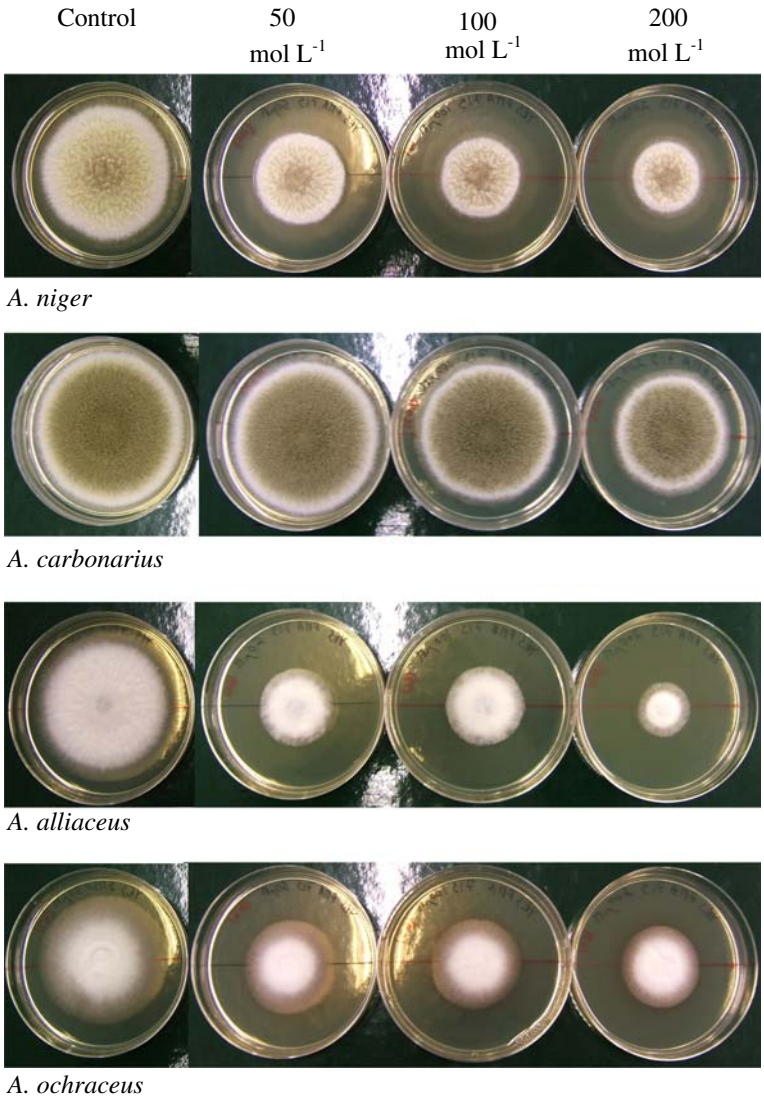


Fig. 1 Effect of 50, 100 and 200 μmol L^{-1} of compound **3a** on the mycelia growth of tested fungi after 4 days of incubation at 25 °C

Table 3 The 50% effective concentration (EC_{50}) of compound **3a** for the different *Aspergillus* spp. tested

	EC_{50} [a]	95% CI [b]	r^2 [c]
A. niger	140.1	107.1 to 183.3	0.9578
A. carbonarius	384.2	327.7 to 450.4	0.9782
A. alliaceus	69.1	53.6 to 89.2	0.9620
A. ochraceus	559.1	437.0 to 715.4	0.9784

[a] EC_{50} = concentration of active ingredient (μmol L^{-1}) at which survival was 50% as determined by fitting the experimental data to a four-parameter logistic model (Hill equation) using computer curve-fitting software (Prism 4, GraphPad Software, Inc, San Diego, CA, USA)

[b] 95% Confidence Intervals of fitted EC_{50}

[c] Correlation coefficient of fitted curves

Discussion

Novel chromene derivatives, obtained by new chemistry, were evaluated for their antifungal properties against selected toxigenic fungi. The novelty of these compounds relies on the very simple and straightforward synthesis and on the absence of halogen elements. This latter property makes these compounds more environmental friendly than some commercial fungicides. Furthermore, the chemistry of these compounds allows the preparation of chromene dimers with different radical in C8 (**3a** and **3b**), which could be useful in the manipulation of their activity.

The chromene dimer **3a** was found to be the most effective of the tested compounds. A moderate inhibitory effect was also observed for the analogue structure **3b** but only for the inhibition of ochratoxin A production. No effect was registered for compounds **2a** and **2b**, used as synthetic precursors of the dimeric species **3**. These results suggest that the dimeric structure is essential to the antifungal activity of species **3**. The highly toxic strain of *A. alliaceus* was found to be the most sensitive to compound **3a** since 200 μmol L^{-1} of this compound was sufficient to inhibit its radial growth by 68% and its ochratoxin A production by nearly 94%.

Recently, Bellí and coworkers [3] tested the effect of several commercial fungicides on *A. carbonarius* strains. In their work, concentrations from 0.035 to 4.0 g L^{-1} were used, depending on the product and as recommended by manufacturers. From 26 products tested, only half of them totally inhibited the growth of the tested strains. In our work, 2000 μmol L^{-1} of **3a**, which represents 0.681 g L^{-1}, inhibit all the tested strains. So, the antifungal capacity of **3a** is comparable to some of the commercial products used by Bellí and coworkers [3], leading to the conclusion that **3a** may have success as a fungi-controlling agent for field applications.

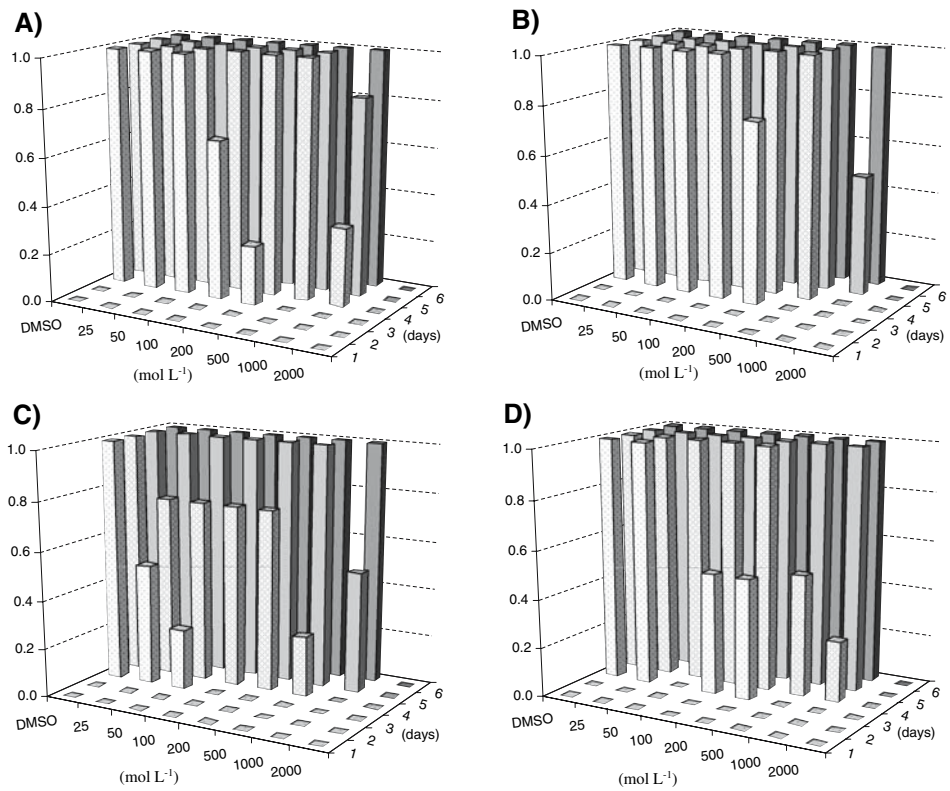


Fig. 2 Presence of visible mycelia when **a** *A. alliaceus*, **b** *A. ochraceus*, **c** *A. carbonarius* and **d** *A. niger* were cultivated in YES medium supplemented with different concentrations of compound **3a** on a 96-well microplate

Table 4 Effect of the compounds in the production of ochratoxin A

Compound	μmol L^{-1}	Ochratoxin A contents (μg/plate)		
		A. carbonarius	*A. alliaceus*	*A. ochraceus*
Control		$16.43 \pm 4.38^{a,b}$	427.04 ± 83.02^{b}	38.29 ± 13.90^{a}
3a	50	21.40 ± 2.26^{a}	290.13 ± 37.99^{c}	45.05 ± 6.52^{a}
	100	22.14 ± 1.44^{a}	229.87 ± 24.62^{d}	53.71 ± 4.86^{a}
	200	19.72 ± 3.84^{a}	25.40 ± 15.68^{e}	43.96 ± 11.19^{a}
2a	200	25.45 ± 5.31^{a}	427.64 ± 12.24^{b}	33.69 ± 8.12^{a}
3b	200	6.38 ± 3.49^{c}	222.73 ± 34.86^{d}	48.76 ± 3.09^{a}
2b	200	$19.04 \pm 5.06^{a,b}$	501.37 ± 6.31^{a}	43.36 ± 12.22^{a}

Values are the mean of three replicates of each ± standard deviation (SD). Data marked with different letters are significantly different from respective control at $P < 0.05$ for the Duncan test

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